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Atty. Docket No.: 1235(203284) PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Habener et al.	Examiner:	M.A. Belyavskyi
Serial No.:	09/963,875		
Filed:	September 26, 2001	Group Art Unit:	1644
Titled:	Stem Cells of the Islets of Langerhans and Their Use In Treating Diabetes Mellitus	Conf. No.:	9674

DECLARATION UNDER 37 CFR 1.131 BY JOEL F. HABENER, M.D.

I declare:

1. I, Joel F. Habener hold an M.D. degree from the University of California, Los Angeles. I received my M.D. degree in 1965. My current position is Associate Physician at the Massachusetts General Hospital and Professor of Medicine at Harvard Medical School. I have held the position of Associate Physician since 1989. I have held the position of Professor of Medicine at Harvard Medical School since 1989. Previously, I held the position of Associate Professor of Medicine at Harvard Medical School from 1975-1988 and also held the position of Howard Hughes Investigator fro 1976-2006. I am an inventor of the above-referenced patent application.

2. I have read the Office Action dated March 10, 2006, filed in the above-referenced patent application and understand that the Examiner has rejected claims 39-41, 43, 74, 77-79, 81, 83 and 85-108 for alleged obviousness over WO 00/09666 or WO 02/086107. It is my understanding that WO 00/09666 can be applied as of it's publication date of February 24, 2000. It is also my understanding that WO 02/086107 can be applied as of it's earliest priority date of April 19, 2001.

3. I initially conceived of the idea that GLP-1 would stimulate neogenesis of beta cells in

June, 1986. This idea was the basis for California Biotechnology Inc.'s (Cal Biochem) establishment of a subsidiary company called Metabolic Biosystems, Inc. (Meta Bio Inc.) for which I served as a consultant for several years (see Exhibit E).

4. In about 1996 my laboratory began to investigate the idea that GLP-1 stimulated neogenesis of beta cells. By July 1997, my laboratory had demonstrated that GLP-1 stimulates the neogenesis of pancreatic beta cells (see Exhibit E).

5. The concept of GLP-1 stimulation of neogenesis of beta cells was discussed with Doris Stoffers and Josephine Egan at the International Congress of Endocrinology/ADA meetings in San Francisco in June 1996 (see Exhibit E).

6. My laboratory began mouse and rat experiments to address whether GLP-1 stimulated neogenesis of beta cells at the time of the ADA meeting in Boston in June 1997. These experiments are described in an NIH grant application filed on February 27, 1997 (see Exhibit F) and in an invention disclosure submitted to MGH CSRL on October 28, 1997 (see Exhibit G). Attached herein are laboratory notes from my Senior Technician, Heather Hermann, from July 1987, that document experiments addressing stimulation of growth of beta cells in vitro with GLP-1 (See Exhibit H).

7. The idea that GLP-1 stimulated the differentiation of new beta cells was premised on the concept that the progenitor cells expressed GLP-1 receptors.

8. In view of the above, the subject matter of claims 39-41, 43, 74, 77-79, 81, 83 and 85-108 that relates to GLP-1R positive human pancreatic stem cells was conceived prior to both the publication date of WO 00/09666 and the earliest priority date of WO 02/086107.

I hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

August 31, 2006
Date

Joel F. Habener

EXHIBIT E

CONFIDENTIAL

Michele Cimbala
December 21, 1998

Conception that Glucagon-Like Peptide-1 (GLP-1) Stimulates the Growth of Pancreatic β-Cells

June 1986: I (Habener) met with Dr. Jeffrey Flier, a co-founder of a then forming new bio-technology company, Metabolic Biosystems, Inc. (MetaBio) to be a subsidiary of California Biotechnology, Inc. (Cal Bio). I discussed with him my ideas that GLP-1 appears to stimulate insulin secretion and may be a growth factor for pancreatic β-cells. Flier and I agreed that this was an exciting possibility. The parent patent on GLP-1 had been filed in May 1986. I was brought in as a consultant for MetaBio with the intention to examine the potential for GLP-1 to stimulate insulin secretion and the growth of new β-cells, and thereby to provide a potential treatment for individuals with diabetes mellitus, a disease known to be a consequence of an inadequate production of insulin by β-cells. We discussed the existing information in the literature indicating that the inadequate production of insulin by the pancreas of diabetic individuals is due to a loss of function of β-cells and/or a reduction in the mass (numbers) of β-cells in the pancreas. Thus, if GLP-1 were to stimulate the growth and production of new β-cells, it would be a very promising potential therapy for the treatment of individuals with diabetes. This conceptualization of the potential for GLP-1 as a treatment of diabetes was clearly in my mind as of June 1986. The concept that the GRP encoded in the anglerfish preproglucagon discovered by us in 1981 and subsequently the orthologous peptide GLP-1 would/may stimulate the growth of β-cells in the pancreas was clearly established in 1981-1982. The whole idea that GLP-1 could be a treatment for individuals with diabetes, and would do so by stimulating the growth of new β-cells in the pancreas, was the major, motivating concept behind the deci-

sion of Cal Bio to establish a subsidiary company, MetaBio, to examine and develop GLP-1 as a therapeutic agent to stimulate insulin production, β-cell growth and functions, and hence β-cell mass in diabetic individuals. In June 1986 we had obtained initial data that GLP-1 stimulates insulin secretion but other hormones, such as glucose-dependent insulinotropic polypeptide (GIP) and cholecystokinin had already been shown to stimulate insulin secretion. Our interest was in whether GLP-1 could stimulate the growth of new β-cells in the pancreas.

7/87-5/ 88 Heather Hermann, a technician in my laboratory, and Habener conduct several studies in an attempt to directly demonstrate that GLP-1 stimulates the growth of pancreatic β-cells. GLP-1 and other proglucagon-derived peptides are added to cultures of insulinoma cells (β-cells) deprived of serum to arrest cell growth. The cells are pulse-labeled with ^{3}H-TdR and emulsion autoradiograms are prepared to examine rates of incorporation of ^{3}H into nuclei of β-cells, as an index of cellular proliferation. The experiments are technically difficult to interpret because the serum deprivation failed to arrest cell division. The concept that GLP-1 stimulates the growth of β-cells prevails. We conclude that the transformed insulinoma cells are not an adequate model to prove that GLP-1 stimulates β-cell growth.

1993-1994 We and other laboratories discover the transcription factor IDX-1 (PDX-1, IPF-1, STF-1) as a major regulator of insulin gene expression and the growth and development of the pancreas and the β-cells. The concept develops in my mind that maybe GLP-1 might stimulate the expression of IDX-1 and thereby may stimulate the neogenesis (new growth) of β-cells in the pancreas. I was then (and still am) excited by the idea that the expression of IDX-1 may be stimulated by cAMP signaling, similar to the transcription factor CREB, that we discovered in 1987. IDX-1, like CREB, had a cAMP-dependent phos-

phorylation site in the active region of the protein. Phosphorylation of CREB at its site is required for activity so I reasoned that IDX-1 activity may likewise depend on phosphorylation. That is, GLP-1 acts on its cellular receptors to stimulate cAMP signaling pathways that may activate the expression of IDX-1, which would then activate the differentiation and growth of β-cells. We recognized in 1985 that GLP-1 activated cAMP formation in β-cells (Drucker et al.). The GLP-1 receptor, cloned by B. Thorens, allowed direct confirmation that GLP-1 acts on a GPCR to stimulate the cAMP signaling pathway. The conception that GLP-1 would cause β-cells to differentiate and to grow was really firmly established and settled in my mind in 1993-1994 because of the discovery of IDX-1, which had cAMP-dependent phosphorylation sites in its structure (Lu et al. 1995). It seemed clear in my mind that GLP-1 indeed stimulates the neogenesis of β-cells.

June 1995 David Zangen presents a paper at the Eur. Acad. Soc. Diabetes describing our collaborative studies with the Joslin Diabetes Center showing that in the regenerating pancreas IDX-1 is highly expressed in the duct cells undergoing neogenesis to form new β-cells. The duct cells that first express IDX-1 go on to express insulin, indicating that the expression of IDX-1 correlates with the differentiation of ductal progenitor cells into the β-cells that produce insulin. The paper is submitted for publication to and sequentially rejected by *Development* and then by *PNAS*. The paper is now in press in *Diabetes*.

9/96-10/96 Habener orders customized matrix assisted delivery (MAD) GLP-1 pellets from Innovative Research of America, Inc. for implantation in to rats and mice to determine whether GLP-1 stimulates β-cell neogenesis, i.e. the differentiation and growth of new β-cells in the pancreas. I first called Innovative Research on 9/20/96 to discuss custom-made GLP-1 pellets. The experimental plan was to maintain GLP-1 pellet-implanted animals for three weeks and then inject the mice/rats with BrdU three hours before their sacrifice and to then examine the pancreata by *in situ* immunocytochemistry

with an antibody to BrdU to give an index of cell division rates. The pancreas sections were also co-stained with antiserum to insulin so that an increase of BrdU staining and insulin staining cells in the pancreatic ducts in response to GLP-1 would indicate that a stimulation of β-cell neogenesis had occurred.

These experiments were intended to provide preliminary studies to support my competing renewal application of my NIH grant DK30834 "Glucagon Biosynthesis and Metabolism."

1/97 The experiments were completed by Doris Stoffers, a postdoctoral fellow in my laboratory, who examined the sections of the pancreata from the mice treated with implanted GLP-1 pellets for 3 weeks with control mice implanted with dummy pellets. For technical reasons the staining with the antiserum to BrdU did not work. The concept that GLP-1 stimulates the neogenesis of β-cells still prevails.

2/28/97 The NIH grant application is submitted. It describes proposed studies with Josephine Egan at the NIA in Baltimore. A letter of intent to collaborate with Habener is provided by Egan and is appended to the grant application. The studies in the proposed collaboration are to determine whether GLP-1 will promote the neogenesis of β-cells in the pancreas.

6/96 At the Endocrine Society Meetings (ICE) held in conjunction with the ADA meetings, Habener introduces Stoffers to Egan and collaborative studies are discussed to determine whether GLP-1 may stimulate β-cell neogenesis and the growth of new β-cells in the pancreas. (There are some additional communications between Egan and me that I have to track down.)

3/97 Stoffers and Egan meet over lunch at the MEEI cafeteria to discuss concrete plans of collaborative experiments to determine the effectiveness of GLP-1 to stimulate the neogenesis of β-cells in mice. I was out of town and could not attend this meeting.

6/97 As agreed upon in the collaborative plan of 3/97 Egan brings the mice that were infused with GLP-1 by subcutaneous osmopumps and mice that had been injected daily with a long-acting GLP-1 agonist, exendin-4, at the NIA in Baltimore, to Boston the week of the annual meeting of the American Diabetes Association, held in Boston, June 1997. On Saturday, June 21, 1997, the mice are sacrificed in my laboratory (Laboratory of Molecular Endocrinology) at the MGH. The pancreata are obtained from the mice for analyses by Western immunoblot and *in situ* immunocytochemistry (ICC) using our antisera to IDX-1, insulin, and other islet hormones.

7/97 Stoffers and I establish that GLP-1(7-36) and exendin-4 both stimulate the expression of IDX-1 in the pancreas on Western immunoblots. Together we view images of the ICC on the computer screen and mutually agree that it appears that the pancreata of mice treated with GLP-1 agonists show an increased expression of IDX-1 and insulin in the epithelial cells of the pancreatic ducts. We also quantitate the sizes of the islets in GLP-1-treated vs. placebo-treated mice (done by a Summer Research Student Jeffrey Rhin and show that the GLP-1 has increased islet mass by two-fold. This is exciting because these observations indicate that GLP-1 has indeed stimulated β-cell neogenesis. The concept that GLP-1 can stimulate β-cell neogenesis, the growth of new β-cells, is completed.

8-10/97 Mehboob Hussain, a postdoctoral fellow in my laboratory, treats AR42J cells with the GLP-1 agonist exendin-4 and shows that the treatment (72 hrs) causes an increase in the expression of IDX-1 and the expression of insulin in these cells. These

findings clearly indicate that GLP-1 can convert AR42J cells to β-cells that produce insulin. The background rationale for doing these experiments is that the AR42J cells were derived from a rat pancreatic carcinoma of ductal origin many years ago. The workers in the exocrine pancreas research have defined the AR42J cells as "amphicrine" cells because they have latent properties of both exocrine and endocrine pancreas cells. The addition of glucocorticoids, such as dexamethasone to AR42J cells converts them to an exocrine phenotype as the cells express amylase, chymotrypsin and other markers of exocrine pancreas cells in response to dexamethasone. Yet AR42J cells are electrically excitable, as are pancreatic endocrine cells. Then in 1995-96 several laboratories showed that treatment of AR42J cells with certain growth factors, such as betacellulin, TGFβ, activin A, hepatic growth factor could convert the cells to an endocrine phenotype that expresses insulin, like β-cells do.

We reasoned that if GLP-1 induces IDX-1 it (GLP-1) may also induce expression of insulin in AR42J cells in response to treatment with GLP-1 agonists. Thus the concept that GLP-1 stimulates pancreatic duct cells to turn on the expression of insulin, and thereby stimulates β-cell neogenesis, is completed again. The data obtained by Hussain are given in the preliminary results section of my resubmission of the amended NIH grant application DK30834 on 10/30/97.

6-7/97 I (Habener) call Egan and inform her of our promising and exciting results. Namely, it looks as though GLP-1 indeed stimulates β-cell neogenesis.

8/97 Egan comes to MGH and meets with Stoffers and me (Habener). We view the data together in the Wellman 3 conference room in the Laboratory of Molecular Endocrinology at the MGH. The computer images of the immunocytochemical staining of the pancreata from GLP-1-treated and saline placebo-treated mice are examined together.

Stoffers and I point out to Egan the evidence for neogenesis stimulated by GLP-1 treatment. Egan agrees that it certainly appears from these experiments that GLP-1 stimulates the neogenesis of β-cells because the enhanced expression of IDX-1 and insulin in the pancreatic ducts of the GLP-1-treated mice is much more pronounced than is seen in the ducts of the saline placebo-treated mice.

4/28/98 Egan gives a seminar at Endocrine Grand Rounds at the MGH. Egan shows data of the results of administration of GLP-1 agonists to mice and the treatment of AR42J cells with GLP-1 agonists (exendin-4). The data demonstrate that GLP-1 agonists stimulate the neogenesis of β-cells. This is of concern, because the data were presented by Egan as though done independently of us. The data were presented in a non-collaborative manner, as if all of the data originated from NIA without collaboration with MGH. It is also interesting that the reason Egan was invited to give Endocrine Grand Rounds is that Elahi asked me to invite her because she was up for promotion to a fulltime position at NIA, a promotion that requires evidence of independence in research, and that having been invited to give a seminar at MGH would look good on Egan's CV and may help obtain the promotion. So I had invited Egan to give this seminar sometime in July or August 1997.

Based on data presented by Egan at Endocrine Grand Rounds, Hussain indicates a loss of interest in continuing experiments of GLP-1 in AR42J cells that will only serve to duplicate Egan's experiments. We understand that the AR42J cell experiments presented by Egan at Endocrine Grand Rounds are in press in the *Journal of Clinical Investigation*.

Concept that the transcription factor IDX-1 (PDX-1/STF-1/IPF-1) is instrumental in the regulation of insulin expression and required for the development of the pancreas (β-cells)

Prior to 1994 it was known that GLP-1 stimulates the transcription of the insulin gene, enhances production of insulin in β-cells, and stimulates secretion of insulin, all in a glucose-dependent manner.

1993-1994 (dates depending on how long it took to get papers revised and accepted). The Laboratories of Habener, Montminy, and Edlund reported simultaneously that the homeodomain transcription factor IDX-1 is islet cell-specific and stimulates the expression of the insulin gene.

1994 Mice rendered nullizygous for *idx-1* are born without a pancreas--the pancreas fails to develop, pancreatic agenesis. Thus IDX-1 is required for not only regulation of transcriptional expression of the insulin gene but also for pancreas development. Stoffers joins my laboratory to do her research training. We learn about a child in Virginia born without a pancreas (pancreatic agenesis). Stoffers and I agree to examine the possibility that the child without a pancreas may be nullizygous for IDX-1. This turns out to be true. The child is homozygous for an inactivating mutation in the *idx-1* gene. We then learn that the child belongs to a very large extended family. The father and mother of the child are obviously hemizygous for IDX-1. The father was diagnosed as having diabetes at age 17. The mother also has diabetes. Examination of the extended family establishes that all carriers of the mutation in the *idx-1* gene have diabetes. Thus, haploinsufficiency in IDX-1 causes diabetes, and the diabetes is due to a lack of insulin production and secretion.

Therefore Habener reasons that if insufficiency in IDX-1 expression causes diabetes, and absence of IDX-1 arrests pancreas development, and that it is known that IDX-1 ex-

pression is restricted to β-cells of the adult pancreas that produce insulin, then IDX-1 may be important in the development of β-cells in the adult pancreas.

Further, and key to the conceptualization, is that GLP-1 stimulates insulin gene expression. Therefore GLP-1 may stimulate IDX-1 expression, and IDX-1 expression so stimulated may stimulate insulin expression. This is an important concept *because* the process of β-cell neogenesis, that is the formation of new β-cells by their differentiation from pleuripotential, or precursor cells in the pancreatic ducts, is believed (by me at least) to recapitulate the ontological development of the pancreas. It is well known that during embryonic development the pancreas is derived by the differentiation of gut endodermal epithelial cells that become the ducts of the pancreas and then give rise to the exocrine and endocrine pancreas (Islets of Langerhans).

1997 It is shown that mice hemizygous for IDX-1 get diabetes at 4-6 months of age. Also CreLox conditional knockouts of the *idx-1* gene get diabetes at 3-6 month of age. Thus in humans (discovered by us) and in mice, loss of *idx-1* expression causes diabetes. The diabetes is due to a reduction in β-cell mass and insulin production. GLP-1 is known to stimulate insulin production.

Importantly, it is well known (early 1990s) that GLP-1 binds to receptors on β-cells and stimulates the formation of cAMP. GLP-1 increases the levels of cAMP in these cells.

1997-1998 What controls the pancreas to develop from a small defined segment of the gut tube during early embryonic development? It was known that the pancreas develops as an evagination of the gut tube at e9.5 from an area of specialized prepatterned endodermal epithelium. Now we know that this specialized region of the gut tube must express IDX-1 and also must not express the important developmental signaling molecule

Sonic Hedgehog (SHH). Given these two circumstances in the gut tube, expression of IDX-1 and repression of SHH, a pancreas will form.

Importantly, it is known that cAMP signaling is a potent antagonist to SHH signaling.

Therefore, I believe that GLP-1, by acting on receptors in pancreatic duct cells and generating cAMP in the adult pancreas, suppresses SHH, and activates IDX-1 expression, and thereby is the mechanism by which GLP-1 stimulates the differentiation of duct cells into β-cells, so-called neogenesis of β-cells. This process of neogenesis of β-cells in the adult pancreas is a recapitulation, a replay, of the embryonic development of the pancreas and of β-cells.

For consideration is my Summary

The concept that GLP-1 stimulates the growth of new β-cells was established in 1986 when it was discussed with Flier as a component for development by the newly formed company, Metabolic BioSystems, Inc. (Meta Bio). There should be letters, records at Scios to document this. Note that in 1986 the company was California Biotechnology, Inc. (Cal Bio) that then became Scios, then Scios Nova, then back to Scios again. Meta Bio was a subsidiary of Cal Bio.

The completion of the concept that GLP-1 stimulates the neogenesis of pancreatic β-cells probably occurred in 7/97.

EXHIBIT F

For Research Office Use:

Spec. Funds ___ C.S. Book ___
Database ___ OTA ___
SHS/SAC ___ Acc. ___

ACC# _____

: this a Clinical Trial? [ ]Yes [ ]No Will it take place in Patient Areas? [ ]Yes [ ]No

H Committee on Research (COR) Research Proposal Coversheet (Instructions are on the back)

Joel F. Habener, M.D.
UNCIPAL INVESTIGATOR (Name, Degree/s Held)

Chief, Laboratory of Molecular Endocrinology
MGH Title

Medicine/Molec. Endocrinol. | Wellman 320 | 6-5190
EPARTMENT/SERVICE and UNIT | ADDRESS (Building/Floor/Room) | Telephone #

GLUCAGON BIOSYNTHESIS & METABOLSIM
roject Title

ENCY NIH NIDDK Agency Application # R01 DK30834

gency
eadline March 1, 1997 Agency Type: [x]Government [ ]Foundation [ ]Industry

lease Indicate: [ ]New [x]Competing Continuation [ ]Non-competing Continuation [ ]Supplemer

; this a RESUBMISSION of A New or Competing Application? [ ]Yes [x]No

PE: [x]Grant [ ]FIRST [ ]RCDA [ ]Other Type
[ ]Contract [ ]CIA [ ]Training Grant
[ ]Subcontract [ ]CIDA [ ]Fellowship

lease check here if the attached is an industrial contract [ ]

PPLICANT ORGANIZATION: [x]MGH [ ]HMS Other Institution _____

ates: 01/01/98 to 12/31/98 (This 12-month Period) 01/01/98 to 12/31/02 (Entire project period)

OTAL DIRECT COSTS this 12-month period $ 138,136 INDIRECT COST Rate 71%

LEASE INDICATE:

uman Study(s)	[ ]Yes	[ ]No	SHS#/ETC 93-4294
rug Study(s)	[ ]Yes	[ ]No	
nimal Study(s)	[x]Yes	[ ]No	
adiation/Isotope Use	[x]Yes	[ ]No	RAD/IS Approval Date 3/31/97
iohazard(s)	[ ]Yes	[ ]No	
ecombinant DNA	[ ]Yes	[ ]No	
se of MGH NMR Facility	[ ]Yes	[ ]No	
se of MGH Cyclotron Facility	[ ]Yes	[ ]No	

EQUIRED SIGNATURES:
rincipal Investigator Joel F Habener Date 2/14/97

hief of Department/Service _____ Date _____

DDITIONAL SIGNATURE:
.G. Chief of Staff,
hriners/Unit Chief, Etc. _____ Date _____

MASSACHUSETTS GENERAL HOSPITAL

Research Affairs
Bartlett Hall 3, Boston, MA 02114
617/726-3651 FAX: 617/726-2796

PHS Certification Requirements - Conflicts of Interest

As of October 1, 1995, the NIH and NSF require all Principal Investigators, Co-Investigators, and all others responsible for the design, conduct, or reporting of the research (herein referred to as "Investigators") to disclose potential conflicts of interest between personal or family financial involvements and the research proposed in the grant application as submitted. These new requirements are detailed in 42CFR part 50, which is available in the Research Administration office.

To summarize the requirements, each Investigator identified in a grant application must disclose to the Institution any "Significant Financial Interests" (i) that would reasonably appear to be affected by the research for which the funding is sought, and (ii) in entities whose financial interests would reasonably appear to be affected by the research. "Significant Financial Interest" means any salary or other payment for services, royalties, and any other payments from a company that in the aggregate exceed $10,000 per year, and any equity interest that exceeds $10,000 or 5% ownership in an entity. The "Significant Financial Interest" includes the aggregate interests held by the Investigator and his/her spouse and dependent children as well. Any conflict of interest must be managed, reduced, or eliminated, as determined by the Institution in accordance with its policy, before a grant can be activated.

All Institution researchers now are required to disclose annually to the Institution and the Harvard Medical School all potential conflicts of interest as defined by the Institution and HMS. The new requirement for disclosure with respect to PHS funding of research now is a part of the Institution and HMS disclosure requirements. Therefore, such disclosures must be included during the annual reporting.

To make possible the submission of a grant application, the PI must insure that all Investigators proposed as participants in the grant disclose any Significant Financial Interest, or certify that they have no such Significant Financial Interest, when the grant application is submitted to the Administration for institutional approval. This form is designed to facilitate such disclosure.

Investigator Name: Joel F. Habener, M.D.

Title of Grant Application: Glucagon Biosynthesis & Metabolism

I hereby certify that I (including my spouse and dependent children):

☒ have no Significant Financial Interest as defined by NIH/NSF policies

☐ have Significant Financial Interest as defined by NIH/NSF policies as follows:
(n.b. - If you have not disclosed this Significant Financial Interest to Harvard Medical School with respect to your activities as a faculty member at that institution, you must complete the HMS form with this information and include that form in a separate, sealed envelope with this grant application.)

- Employment, Consultant, or Royalty: (income from which, in the aggregate [including that to my spouse and dependent children], exceeds $10,000 per year):

_____

_____

_____

- Financial Interests, including Equity: (which exceeds $10,000 or 5% share of the entity [including that held by my spouse and dependent children]):

_____

_____

_____

Joel F. Habener
Signed - Investigator

February 27, 1997
Date

(RA-9/95)

AA

Form Approved Through 9/30
OMB No. 0925-00

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK—FOR PHS USE ONLY.		
Type	Activity	Number
Review Group	Formerly	
Council/Board *(Month, Year)*		Date Received

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*
GLUCAGON BIOSYNTHESIS & METABOLISM

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT [X] NO [ ] YES *(If "Yes," state number and title)*
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME *(Last, first, middle)*
Habener, Joel Francis

3b. DEGREE(S)
M.D.

3c. SOCIAL SECURITY NO.
572-50-7801

3d. POSITION TITLE
Professor of Medicine

3e. MAILING ADDRESS *(Street, city, state, zip code)*
Laboratory of Molecular Endocrin.
Massachusetts General Hospital
55 Fruit Street - WEL320
Boston, MA 02114

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Medicine

3g. MAJOR SUBDIVISION
Molecular Endocrinology

3h. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (617) 726-5190
FAX: (617) 726-6954

E-MAIL ADDRESS:
habenerj@al.mgh.harvard.edu

4. HUMAN SUBJECTS [X] No [ ] Yes
4a. If "Yes," Exemption no. or IRB approval date
[ ] Full IRB or [ ] Expedited Review
4b. Assurance of compliance no.
M1331-01

5. VERTEBRATE ANIMALS [ ] No [X] Yes
5a. If "Yes," IACUC approval date
2/19/97
5b. Animal welfare assurance no.
A3596-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
01/01/98	12/31/02	138,136	228,340	748,192	1,236,769

9. APPLICANT ORGANIZATION
Name: Massachusetts General Hospital
Address: The General Hospital Corp.
55 Fruit Street
Boston, MA 02114

10. TYPE OF ORGANIZATION
Public: → [ ] Federal [ ] State [ ] Local
Private: → [X] Private Nonprofit
Forprofit: → [ ] General [ ] Small Business

11. ORGANIZATIONAL COMPONENT CODE: 30

12. ENTITY IDENTIFICATION NUMBER: 1042697983A1

Congressional District: 9

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: Marcia L. Smith
Title: Dir., Proposal/Award Management
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14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Marcia L. Smith
Title: Dir., Proposal/Award Management
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Boston, MA 02114

Phone: (617) 726-3651
FAX: (617) 726-2796

E-Mail Address: smith@helix.mgh.harvard.edu

15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI / PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*
Joel F. Habener
DATE: 2/27/97

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. *(In ink. "Per" signature not acceptable.)*
per Marcia L. Smith
DATE: 2/27/97

GLOSSARY of Abbreviations

Brn-4	pou-specific homeodomain protein Brain-4
CCK	Cholecystokinin
CHOP	c/EBP homologous protein
CRE	cAMP-response element
CREB	Cyclic AMP response element binding protein
CRH	Corticotropin-releasing hormone
GHRH	Growth hormone-releasing hormone
GIP	Gastric inhibitory peptide
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
IDX-1	Islet duodenal homeobox protein
K-ATP	ATP-sensitive K^+ channels
MPF	Major proglucagon fragment
MyoD	Muscle specific transcription factor
NIDDM	Non insulin dependent diabetes mellitus
PACAP	pituitary adenylyl cyclase-activating protein
Pan-1	Transcription factor E47
PTH	Parathyroid hormone
SUR	Sulfonylurea receptor
VDCC	Voltage-dependent Ca^{2+} channels
VIP	Vasoactive intestinal peptide

DESCRIPTION. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

The disease non insulin dependent mellitus (NIDDM) is increasing by epidemic proportions in developed countries throughout the world. There are an estimated 100 million individuals with diabetes and an equal number who have not yet been diagnosed. One important manifestation of NIDDM is impaired and/or dysregulated secretion of the hormones insulin and glucagon in conjunction with impaired insulin sensitivity (insulin resistance). Glucagon is a catabolic hormone whose physiological actions are counter to those of the anabolic hormone, insulin. Hyperglucagonemia is a common manifestation of diabetes, increases hepatic glucose output, and worsens hyperglycemia. The overall hypothesis being tested in these studies is that the regulation of the expression of the glucagon gene is critically important during the switch from fasting (catabolic) to the fed (anabolic) state. The glucagon gene is expressed in both the pancreas and the intestine. Remarkably, by mechanisms of alternative post-translational processing of proglucagon, the pancreas produces the bioactive peptide glucagon, the anti-insulin hormone important in the fasting state to maintain blood glucose levels. In the intestine the bioactive hormone produced is glucagon-like peptide-I (GLP-I), an incretin hormone that has potent insulinotropic actions on β-cells of the pancreas, satiety actions on the hypothalamus, and possible peripheral actions on adipose and skeletal muscle to enhance glucose uptake and on liver to inhibit glucose output. It is proposed that: 1) During fasting, glucagon gene expression is tonically elevated due to the low insulin and glucose levels and high neuroadrenergic inputs likely mediated by cAMP-dependent signaling pathways. 2) During feeding, oral nutrients induce intestinal L-cells to release the insulinotropic hormone GLP-I that activates specific cAMP-coupled receptors on pancreatic β-cells and, synergetically with glucose, stimulates insulin and represses glucagon release and production, respectively. We propose to continue our investigations of the mechanisms involved in the transcriptional expression of the glucagon gene. The aims are to: (1) examine the potential role of the pou-specific homeodomain protein Brain-4 in the α-cell-specific expression of the proglucagon gene and as a possible factor in α-cell development; (2) isolate, identify, and characterize the peripheral GLP-1 receptor expressed on adipocytes. We propose to clone the receptor from a 3T3-L1 cell cDNA expression library, prepare stable cell lines expressing the receptor, characterize the hierarchy of peptide hormone binding and the coupling to signal transduction pathways, and investigate the potential role of the receptor in diabetes; (3) examine the potential properties of GLP-1 to enhance growth and to inhibit apoptosis of pancreatic β-cells. The importance of hormones encoded by the glucagon gene in the maintenance of glucose homeostasis, and their potential relevance to the pathogenesis of NIDDM, provides interest in learning more about the controlling factors involved in the expression of the gene.

PERFORMANCE SITE(S) *(organization, city, state)*

Massachusetts General Hospital
Boston, MA

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Joel F. Habener, M.D.	Massachusetts General Hospital	P.I.
Colin A. Leech, Ph.D.	Massachusetts General Hospital	Research Assoc
Karen S. McManus	Massachusetts General Hospital	Technician

Type the name of the principal investigator/program director at the top of each printed page and each continuation page. (For type specifications, see instructions on page 6.)

RESEARCH GRANT

TABLE OF CONTENTS

Research Plan

*Type density and type size of the entire application must conform to limits provided in instructions on page 6.

Appendix (*Five collated sets. No page numbering necessary for Appendix.*)

[X] Check if Appendix is included

Number of publications and manuscripts accepted or submitted for publication (*not to exceed 10*) 10

Other items (*list*):

the peripheral GLP-1R no longer generates cAMP, but rather acts through Ca^{2+} or PI or other signaling pathways to stimulate lipogenesis and not lipolysis.

Several lines of investigations of the novel GLP-1 receptor may be productive. One would be to ask whether this receptor is also expressed in the liver and muscle, or even brain or islet cells, and to determine the tissue distribution of the receptor by using approaches of RT-PCR of tissue extracts, RNase protection assays. *in situ* histohybridization, and immunocytochemistry and Western immunoblots, such as we did for the pancreatic-type GLP-1R described in our paper **P5**. One of the first things that we will do is to prepare an antiserum to the novel GLP-1R using a synthetic peptide to a sequence estimated to be just carboxy-proximal to the N-terminal signal sequence of the receptor.

It would be interesting to investigate the ion channel activities and effects on $[Ca^{2+}]_i$ of the ligand-activated receptor in single cells using the patch clamp electrophysiological approaches and fura-2 dual-wave length calcium imaging. Ion channel activities could be examined in the L-6 myocytes because they are electrically excitable cells.

If GLP-1 enhances insulin stimulated glucose uptake in cells (3T3-1 adipocytes) as reported by Egan et al. [55], it may do so by enhancing the translocation of glucose transporter-4 to the plasma membrane. This can be examined by measuring the change in capacitance of the cell in response to GLP-1.

Another potentially interesting line of investigation might be to determine whether the novel peripheral GLP-1R is upregulated or downregulated in animal models of diabetes. Circulating GLP-1 levels are reported elevated in diabetic rats, as well as in NIDDM subjects [62, 63]. Therefore, increased circulating GLP-1 may contribute to obesity in diabetic subjects. If the peripheral GLP-1R is coupled to signal transduction pathways different from the cAMP pathway to which the pancreatic GLP-1R is coupled it may not desensitize. One might speculate that eventual administration of GLP-1 to NIDDM subjects might enhance peripheral glucose utilization, in addition to stimulating insulin secretion and inhibiting feeding behavior.

Another line of investigation might be to examine the cross-talk between the leptin cytokine and the GLP-1 signaling pathways. Leptin is the obesity (starvation) hormone made in fat that inhibits feeding, increases energy expenditure and reproductive processes. Leptin also has receptors on adipocytes (recent Keystone meeting) and inhibits insulin-mediated glucose uptake in skeletal muscle (recent Keystone meetings on diabetes and obesity). Cyclic AMP signaling is well recognized to antagonize cytokine signaling. Since we have recently found that leptin inhibits insulin secretion by hyperpolarizing β-cells via opening ATP-sensitive K^+ (K^+-ATP) channels. and the leptin receptor (Ob-Rb long form) is coupled to the Jak/STAT and possibly MEKK/ERK cytokine pathways, GLP-1 signaling may act on K-ATP (Kir6.2 or SUR) or on Ob-Rb, or on Jak or STAT to antagonize leptin inhibition. Such a cross-talk between GLP-1 signaling and leptin signaling may explain how insulin can be secreted during meals when incretins such as GLP-1 are released and overcome the inhibitory actions of leptin on insulin secretion. Further avenues of investigation of the peripheral GLP-1R would be to examine the gene structure and determine whether alternative exon-splicing occurs and modifies receptor activity, examine the promoter and identify transacting factors that may be expressed during the differentiation of 3T3-L1 adipoblasts to adipocytes, thereby activating the transcription of the receptor gene. The physiologic importance of the GLP-1R can be tested by producing mice with a targeted disruption of the gene, as has been done for the pancreatic GLP-1R by Drucker et al., resulting in a phenotype of glucose intolerance. Double transgenic mice may be created by crossing mice with knockouts of the peripheral GLP-1R to mice with knockouts of the pancreatic GLP-1R.

3. Investigations of potential actions of glucagon-like peptide-1 (GLP-1) on β-cell differentiation, proliferation, and apoptosis

The hypothesis to be examined is that the long-term administration of GLP-1 may enhance β-cell mass. We plan to administer GLP-1 long-term to streptozotocin diabetic mouse and rat models and to directly test the effects of GLP-1 on β-cell proliferation, differentiation, and apoptosis and thereby to establish whether or not GLP-1 may be a determinant of β-cell mass. Three experimental approaches are proposed: (1) The aged diabetic Wister rat model (in collaboration with J. Egan, NIH Age Institute); (2) The regenerating pancreas model (in collaboration with Drs. Gordon Weir and Susan Bonner-Weir, Joslin Research Laboratories) and (3) The transgenic IDX-1 promoter-LacZ reporter mice that we have generated in our laboratory (Stoffers et al., submitted for publication). Before describing the details of the experimental approaches, some background and rationale for justifying the undertaking of these experiments is required.

The actions of GLP-1 on its receptor, at least in β-cells, generates high cellular levels of cAMP. Cyclic AMP is well known to stimulate the proliferation of many different cell types and to promote differentiation of other cell types [reviewed in 120]. Phosphorylated CREB also activates the transcription of the BCL-2 gene, increases cellular levels of BCL-2, and rescues apoptosis in B-lymphocytes [126]. BCL-2, the mammalian homologue of the nematode protein Ced-9 (c. elegans death protein-9), is a potent universal inhibitor of apoptosis [127, 128] (**Fig. 16**). BCL-2 is a 26 kDa protein, located in membranes of the mitochondria and endoplasmic reticulum, that is believed to protect against apoptosis by decreasing the net cellular generation of reactive oxygen species (ROS)

and lipid peroxidation. Notably, glycation end products are implicated in the generation of ROS and chronic hyperglycemia enhances the formation of glycation end-products. Furthermore, glycation-dependent ROS appear to mediate the suppression of insulin promoter activity in hamster insulinoma (HIT) cells [129, 130]. A number of BCL-2 homologs have been identified, including BCL-X_L, and BAG-1, and Bad, which promote apoptosis. Current evidence suggests that the ratios of these anti-to pro-apoptotic proteins may play a regulatory role in apoptosis [131].

It seems plausible, therefore, that long-term administration of GLP-1 to rat or mouse models of reduced β-cell mass and impaired β-cell function may either stimulate β-cell proliferation/differentiation and/or inhibit apoptosis (**Fig. 17**). If GLP-1 does have any of these actions, their demonstration would be relevant to the rational for the long-term treatment of diabetic subjects, not only those with NIDDM, but also possibly insulin-dependent diabetes mellitus (IDDM) type I, juvenile diabetes in which the β-cell mass is severely reduced but in which progenitor β-cells located in the pancreatic ducts remain viable. To date, few long-term studies of GLP-1 administration have been done, particularly studies in which the parameters of β-cell proliferation, differentiation, and apoptosis have been examined. As described in C. Progress Report/Preliminary Studies, 48-hr infusions of GLP-1 to aged diabetic (23 month old) rats results in a marked stimulation of insulin secretion and production and in β-cell proliferation (J. Egan, NIH Aging Institute, submitted for publication). We are planning to collaborate with Dr. Egan to examine the effects of the GLP-1 infusions on the expression of IDX-1 in the β-cells (see letter of intent to collaborate, Appendix). It appears that the transcription factor IDX-1 is a positive regulator of insulin gene expression and is involved in pancreatic development. That IDX-1 is involved in the differentiation of progenitor pancreatic duct cells into insulin-producing β-cells is supported by a collaborative study done with Dr. Gordon Weir at the Joslin Research laboratories (Zangen et al., submitted for publication) using the regenerating pancreas rat model following partial pancreatectomy. During the first days following 90% pancreatectomy, the pancreatic remnant undergoes an intense proliferative phase of the ductal cells followed by the expression of insulin and glucagon as the ductal cells differentiate into endocrine cells. In essence, this regenerating pancreas model recapitulates the ontological development of the pancreas. We have observed a marked increase in IDX-1 and insulin gene expression between days 2 and 3, corresponding to the transition from the proliferative to the differentiation phase of regeneration. These findings support the idea that IDX-1 is involved in the differentiation of ductal progenitor β-cells to mature β-cells. Further, these initial findings raise the possibility that the regenerating pancreas model may be a means to test the hypothesis that GLP-1 and cAMP signaling may stimulate the process of the differentiation of ductal progenitors of β-cells to mature insulin-producing cells.

Experimental methods

Long-term administration of GLP-1 to rats and mice

A proven effective method to deliver hormones to rats and mice is to use implanted mini osmopumps (Alzet. Inc.). However, the pumps do get plugged and fail to deliver the hormone, for technical reasons (our observations). As an alternative to osmopumps, we have been experimenting with matrix assisted delivery pellets, prepared by Innovative Research America, Inc. We provided them with 20 mg of GLP-1(7-37) which they formulated into time-release pellets for our use. The pellets are inserted under the skin of rats or mice with a trochar. The pellets deliver GLP-1 for up to 21 days and achieve blood levels of 20-50 pM equivalent to prandial levels. Placebo pellets are provided to serve as controls. However, we have not yet thoroughly evaluated matrix-assisted delivery pellets for delivery rates and pharmacodynamics. We do have preliminary data on osmopumps that are encouraging and they can be used if matrix pellets don't work. We tested both 0.2 ml and 2.0 ml 7-day delivery pumps implanted subcutaneously in the nape of 500 gm rats. The GLP-1(7-37) solutions contained 2 mg (0.2 ml) and 10 mg (2.0 ml). At the end of 5 days of GLP-1 administration, the animals were sacrificed. Plasma GLP-1 levels achieved were 4 ng/ml and 0.8 ng/ml for the 2.0 ml and 0.2 ml pumps, respectively (T1/2 3-4 min, MCR 11 to 14 ml.min). This rangefinder study allows us to calculate the desired dose of GLP for administration. Normal basal and prandial GLP-1 levels are 2 and 10 pM and 10 and 60 pM for the 7-37 and 7-36amide isopeptides, respectively. We would aim for infusion plasma levels of 50-100 pM (150-300 pg/ml). Thus, the smaller pumps (0.2 ml) containing 0.5-1.0 mg GLP-1(7-37) can be used.

Creation of streptozotocin diabetic mice and rats

Streptozotocin is a relatively specific β-cell toxin, believed to act by specifically compromising the NADH-ryanodine, cyclic ADP ribose metabolic system unique to β-cells. Streptozotocin can be administered at low doses to create "NIDDM" models and at higher doses to create "IDDM" models of diabetes. The protocols for generating these mouse and rat models of diabetes are well documented [132, 133].

Experimental parameters to be evaluated

The extent of impaired glucose tolerance (IGT) or diabetes in response to streptozotocin will be monitored by measuring urine and plasma glucose levels, and by oral glucose tolerance tests [134] in which we will measure blood glucose, insulin, and GLP-1 levels.

(i) The cAMP antagonist (Rp cAMPs) also evoked the current, suggesting that the cAMP actions were not mediated by PKA, but perhaps by the binding of cAMP to a protein, perhaps the Na/Ca-NS. (ii) The elicited current is voltage independent and not inhibited by VDCC blockers such as nifedipine and veraparmil or by clamping the voltage at -70 to -100 mV at which VDCCs cannot open. (iii) The activation of the current is also totally dependent on extracellular Na^+ and Ca^+ chelators BAPTA-AM or EGTA, suggesting the participation of a Na^+/Ca^+ exchanger and movement of Ca^{2+} from intracellular stores, respectively. **Figure 9** provides a model with which to evaluate these diverse actions of GLP-1. Receptor occupancy by GLP-1 activates $G_{s\alpha}$ proteins and stimulates adenylyl cyclase, thereby accelerating conversion of ATP to cAMP. We propose that this catalytic process is dependent on extracellular Na^+ and that the subsequent binding of cAMP to cyclic nucleotide-regulated non-selective cation channels (or a protein closely associated with the channel) results in channel activation, thereby generating I_{cAMP}. Activation of these channels by cAMP is also proposed to require intracellular Ca^{2+}. The rise of $[Ca^{2+}]_i$ which accompanies I_{cAMP} is achieved by stimulation of at least two distinct Ca^{2+} signaling pathways. First, the membrane depolarization that is a direct consequence of I_{cAMP} results in activation of VDCCs, thereby raising $[Ca^{2+}]_i$. Second, a rise of $[Ca^{2+}]_i$ is observed even under conditions in which the membrane potential is voltage-clamped at values (-100 to -70 mV) negative to the activation threshold of VDCCs. Although the nature of this additional rise of $[Ca^{2+}]_i$ remains to be determined, it may signify the mobilization of Ca^{2+} from intracellular stores, as well as Ca^{2+} influx via nonselective cation channels and/or membrane transporters (see below). Acting in concert, these Ca^{2+} signaling pathways are proposed to contribute to the stimulatory actions of GLP-1 on insulin secretion from β-cells.

From a functional standpoint, the ability of GLP-1 to raise $[Ca^{2+}]_i$ through activation of a signaling system and not involving effects on I_KATP has at least one important ramification. GLP-1 augments insulin secretion in non-insulin-dependent diabetics, even under conditions in which the sulfonylurea drugs such as glyburide (which inhibits I_KATP) fail to stimulate insulin secretion (sulfonylurea failure). This observation suggests that one therapeutic advantage of GLP-1 relative to that of sulfonylureas in the treatment of non-insulin-dependent diabetes is that GLP-1 triggers a rise of $[Ca^{2+}]_i$, insulin secretion, and a lowering of blood glucose, even under conditions in which sulfonylurea receptors and ATP-sensitive potassium channels no longer play a dominant role in the regulation of β-cell stimulus-secretion coupling. Therefore, activation of I_{cAMP} by GLP-1 may serve as a reserve mechanism of action, one that complements its previously reported inhibitory effects on I_KATP. This would then explain why the glucagon-like peptides retain their biological activity and augment insulin secretion even under conditions in which sulfonylureas are no longer effective.

We also showed that the voltage independent Na^+/Ca^{2+}-NS channels are involved in the slow oscillations in β-cells (**P8**) and are part of the β-cell depolarization mediated by pituitary adenylyl cyclase-activating protein (PACAP) (**P9**). The spontaneous slow oscillations in cytosolic calcium in β-cells are mediated by voltage-independent channels (**P8**). These observations suggest that the slow oscillations in $[Ca^{2+}]_i$ may serve as important initiators of insulin secretion under conditions in which tight control of glucose homeostasis is necessary such as during the fasting normoglycemic state. It is proposed that PACAP may be important in a neuro-entero-endocrine loop regulating insulin secretion during the transition period from fasting to feeding (**P9**).

Potential trophic effects of GLP-1 on β-cell neogenesis and proliferation

Several lines of evidence suggest the possibility that GLP-1 may have trophic actions on β-cells. GLP-1 is known to stimulate cAMP formation in β-cells and to stimulate insulin secretion, and also insulin biosynthesis. Further, it is known that cAMP is an effective second messenger and stimulates certain cell types to proliferate and in other cell types inhibits proliferation and induces cellular differentiation [120]. Essentially all of the studies of GLP-1 actions conducted thus far have been short term experiments to demonstrate the insulinotropic actions, i.e., to stimulate insulin secretion and to lower blood glucose levels. In preliminary studies with Dr. J. Egan of the Aging Institute 48 hr continuous infusions of GLP-1 have been administered by subcutaneously implanted mini osmopumps to aged rats (23 months old). This particular strain of Wister rats develops hyperinsulinemia and glucose intolerance at about one year and by 18 months develop diabetes akin to NIDDM. The results of initial studies of the 48 hr infusions of GLP-1 to aged rats has provided some provocative results. Insulin secretion and glucose tolerance (oral) in these aged rats, several hours after the termination of the GLP-1 infusion, was normalized to that of the younger 6 months old rats with normal insulin secretion and glucose tolerance. Further, by immunostaining sections of the pancreas and RIA of extracts of pancreas, insulin content increased by 2-fold compared to sham infused control rats. In effect, the 48 hr infusion of GLP-1 converted the glucose/insulin physiology of aged rats with impaired (diabetic) insulin secretion to that of normal young rats. Further, preliminary measurements of the rates of proliferation of β-cells in response to the GLP-1 infusions, using immunostaining of the pancreas with an antiserum to proliferating nuclear antigen (PCNA), suggests a stimulation of proliferation. These preliminary findings suggest the possibility that the long-term administration of GLP-1 may stimulate β-cells neogenesis and/or proliferation and raise the further possibility that the expression of the homeodomain protein IDX-1 or the E47 proteins may be upregulated to account for the increase in insulin production. Much more experimentation is required to support these preliminary findings (See D. Experimental Design and Methods). However, it is tempting to speculate that an eventual long-term treatment of diabetic subjects with GLP-1 may not only stimulate insulin secretion and peripheral glucose utilization, suppress glucagon secretion and hepatic glucose output, but also enhance the formation of β-cells.

12th International Symposium on Regulatory Peptides

Type name, address, and telephone number of authors who should receive correspondence in area C and complete areas A and B. Please be sure to check the appropriate category for your abstract in area B.

A. Important

The principal author affirms that the material herin;
1) will not have been published as an article by September 1998 2) that if human subjects were exposed to risks not required by their medical needs, the study was approved by an appropriate committee or, if no such committee was available and informed consent was needed, it was obtained in accordance with the principles enunciated in "The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research," US Government Printing Office: 1983-381-132:3205, or 3) any animal studies conform with the "Guiding Principle in the Care and Use of Animals" of the American Physiological Society.

Signature of Principal Author

☒ Check if abstract is submitted for the Young Investigator Travel Award

Please check all categories that apply:

- ☐ [illegible]
- ☐ [illegible]kines/Agonists
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- ☐ [illegible] Biosynthesis
- ☐ [illegible]
- ☐ [illegible]
- ☐ [illegible] Regulation of Genes
- ☐ [illegible] Nitric Oxide

Abstract Deadline March 1, 1998

GLP-1 IS A TROPHIC FACTOR.

J. M. Egan, J. Zhou, R. Perfetti, D. Elahi,
NIA, Baltimore, MD.; MGH, Boston, MA.

Glucagon-like-peptide-1 (GLP-1) is a potential candidate for the treatment of type 2 diabetics because it normalizes blood glucose levels. We have shown that GLP-1 increases intraislet insulin content in Wistar rats. This is accompanied by an increase in mRNA of insulin, glucokinase and GLUT2 transporter. There is a 26% increase in pancreatic weight with 5 days of chronic subcutaneous treatment with GLP-1. To examine whether an increase in cell turnover occurs during treatment with GLP-1 or a potent analog, exendin-4, we used proliferation cell nuclear antigen (PCNA) as a marker for proliferation of cells. An increase in PCNA in the acinar portion of the pancreas and in the progenitor pancreatic cells in the ducts was observed. Furthermore there was a progression with the greatest density in PCNA positivity observed in the smallest ducts to the lowest density in the largest ducts. Two days of treatment with GLP-1 (1.5pmol.kg^{-1}.min^{-1}) or daily injection of exendin-4 (0.5 nmol/kg, IP) resulted in glucagon containing cells in the ducts. After 6 days of treatment, positive staining for insulin was observed in the smallest ducts as well as in small nests of cells within the acinar portion of the gland. We conclude that GLP-1/exendin-4, similar to what has been shown for GLP-2 in the intestine, causes differentiation of progenitor pancreatic cells into cells containing glucagon and insulin.

I, [illegible] Elahi certify that Jie Zhou is eligible for the Young Investigator Travel Award.
(Laboratory Director)

C.

Principal Author's Name: Dariush Elahi
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EXHIBIT G

Laboratory of Molecular Endocrinology
Massachusetts General Hospital
Howard Hughes Medical Institute

Wellman Bldg 320, 50 Blossom Street
Boston, MA 02114
Tel. (617) 726-5190; Fax: (617) 726-6954

TO: DAVID GLASS

FROM: JOEL HABENER

DATE: 10/28/97

FAX NO: 6-1668

NO. of Pages: 7
(including this page)

David H:,

This may need to be revisited. Some of the data have been generated with the NOVO Nordisk analog. Other data by/with NIH collaborators.

Joel

Please call if entire transmission is not received or if it is routed incorrectly. Thank you.

MODE = MEMORY TRANSMISSION START=OCT-19 15:58 END=OCT-19 16:02

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Fax

To: MARIE LOSSKY **From:** JOEL HABENER

Fax: 6-1668 **Date:** 10/19/00

Phone: **Pages:** (inclusive)

Re: **CC:**

☐ **Urgent** ☐ **For Review** ☐ **Please Comment** ☐ **Please Reply** ☐ **Please Recycle**

•Comments:

Hi!

This is the original disclosure - Communications. The new findings are that the progenitor cells are the nestin-positive islet-derived progenitor stem cells.

GLP-1 → Islet derived Stem Cells → β-cells
(?IDX-1)

Joel

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Fax: 6-1668 **Date:** 10/19/00

Phone: **Pages:** (inclusive)

Re: **CC:**

☐ **Urgent** ☐ **For Review** ☐ **Please Comment** ☐ **Please Reply** ☐ **Please Recycle**

•Comments:

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From: Lossky, Marie

To: Habener, Joel F

Cc:

Subject: RE: New patent

Sent: 10/18/00 8:04 AM **Importance:** Normal

Joel,
Thanks for the fax. I'll have to get Marv involved in positioning IP protection around this so as not to throw off any of your existing IP portfolios. It would be helpful to us if you would fill out the attached invention disclosure form. In particular, what experimental evidence do you have to support the hypothesis? How does the hypothesis fit in with prevailing thinking in the field?

Also, could you please expand/comment on your proposed use of the GI mice? You mentioned in passing yesterday that you wanted to use these animals in some of your stem cell work, and I am not sure which of the following statements (from Exhibit A, the Research authorized under the Material Transfer Agreement with GI) would be relevant:

1. Analyze GFP expresion during embryonic development and correlate the findings with results from immunohistochemical analyses of PDX protein levels in tissue sections from the same mice.

2. Use the PDX/GFP and INS/LacZ transgenic mice in stides to determine the effects of islet growth factors, such as GLP-1, on PDX and insulin gene expression in adult mice.

3. Look for GFP expression in brain sections from PDX/GFP transgenic mice to corroborate and extend intitial findings of PDX expression in mouse brain.

I should remind you that any patentable inventions or unpatentable results that you obtain using these animals are obligated to GI's parent company, American Home Products. This may not be an opportune time to enter into agreements that tie up pieces of your stem cell story, but of course the decision to do that is ultimately up to you.

Thanks.
Marie

Invention Disclosure Form.ID+NCD.5.00.doc

Marie Lossky, Ph.D.
Industry Agreement Associate
Corporate Sponsored Research and Licensing,
Massachusetts General Hospital
tel: (617) 726-8629
fax: (617) 726-1668

-----Original Message-----

From: Habener, Joel F
Sent: Tuesday, October 17, 2000 6:35 PM
To: Lossky, Marie
Subject: New patent

Hi!
I just faxed the model and the idea of linking the various components of the idea together.
Joel

Disclosure Received: 10/28/97

Disclosure No: _____

MGH INVENTION DISCLOSURE FORM

1. *TITLE OF INVENTION:*

Stimulation of IDX-1 expression and β-cell neogenesis by GLP-1

2. *INVENTOR(S) NAME, TITLE, LAB, DEPT., AND TEL. EXT.:*

Joel F. Habener, MD
Professor of Medicine
Laboratory of Molecular Endocrinology 6 6950

3. *SOURCE OF FUNDS FOR THE RESEARCH WHICH RESULTED IN THE INVENTION:*

A.		Government Grant - Agency and Grant No.	DK 30834, DK 30457
B.		Private Industry - Name	
C.		MGH	
D.		Foundation	
E.		Other - Explain	Howard Hughes Medical Institute

4. *INVENTION DISCLOSURE:* Describe the invention in sufficient detail, using the outline below to convey a clear understanding of the nature, purpose, operation and the physical, chemical biological or electrical characteristics of the invention. Attach sketches, drawings, photos, diagrams or photos, and any pertinent manuscript which described the invention:

A. State in general terms the purpose and object of the invention.

B. Describe the background of the invention and how the invention overcomes problems that existed previously.

(1)

C. Describe the invention in detail, particularly pointing out novel features and critical components. Include sketches, drawings, circuit diagrams. If the invention relates to a new composition of matter, give the structural formulas for all novel compounds, the process for synthesizing or isolating the compounds, all available chemical and physical properties and all test data which show the utility and efficacy of the compounds. A copy or manuscript of a draft including this information will usually be acceptable.

5. *PUBLICATION, SALE OR USE OF THE INVENTION:*

A. Have you described your invention in a publication?

YES		NO	X

B. If YES, give name and date of publication.

C. If NO, what plans do you have for publication in the future?

Prepare a manuscript in 1-2 months

D. Has your invention been used? When and under what circumstances?

GLP-1 is under development by NOVO Nordisk to treat type 2 diabetic subjects

E. Has your invention been offered for sale? When and under what circumstances?

No

6. *COMMERCIAL POSSIBILITIES:* To the extent know, please state whether the invention has significant commercial potential. Is the invention primarily a research tool? How extensively will it be used by the public? Does it appear to have significant commercial potential outside the United States?

It is potentially useful for treatment of diabetes mellitus, estimated 100 million individuals world-wide

7. *HISTORY OF THE INVENTION:*

A. When did you first think of (conceive) the invention?

Date	6/85

B. When did you first disclose your invention to another person?

Date	6/85	To Whom	Heather Hermann

C. When was the first written description or drawing of your invention produced? Please attach photocopy of such written description.

Date	NA

8. *INTERACTIONS WITH THIRD PARTIES:*

A. Have you or any co-inventor(s) listed in (2) above received Biological Materials from any industrial or academic source for use in the research which gave rise to the invention? If so, please list the Material and attach a copy of each such Agreement.

No

B. Have you or any co-inventor(s) listed in (2) above entered into or signed a confidentiality or secrecy Agreement in exchange for receiving any proprietary information from a third party pertaining to the research which gave rise to the invention? If so, please describe briefly the subject or the confidentiality or secrecy Agreement (s) and attach a copy of each such Agreement.

No

INVENTOR(S)' SIGNATURE(S): *DATE*

Joel F. Hobener 10/28/97

WITNESS(ES): Disclosed to and understood by me on:

DATE *SIGNATURE*

4

4. Diabetes Mellitus affects approximately 16 million people in the USA (100 million world-wide). Individuals with type 1 diabetes have lost their ability to produce insulin due to the immune destruction of their pancreatic β-cells, which secrete insulin. Individuals with type 2 diabetes have lost their ability to over-produce insulin to maintain euglycemia in the presence of insulin resistance. In both types of diabetes there is a marked reduction in the mass of β-cells in the pancreas.

It is believed that the endocrine panacæas (β-cells) are derived from progenitor cells in the ducts of the exocrine portion of the pancreas. Transcription factors have been identified that are involved in pancreatic development and the stimulation of insulin gene transcription. The expression of transcription factors is believed to be regulated by growth factors, otherwise known as hormones or morphogens. Glucagon-like peptide-1 is an intestinal hormone that is released in response to feeding and stimulates the β-cells to make and secrete insulin. GLP-1 is under development as a promising potential treatment for type 2 diabetes, because it stimulates the pancreas to make its own insulin and it does not over-stimulate insulin secretion because its actions shut off when the blood sugar drops to dangerous levels. In addition, GLP-1 is now known to control appetite and to induce individuals to loose weight. GLP-1 also augments insulin mediated uptake of glucose by the liver, skeletal muscle and adipose tissue, thereby improving insulin sensitivity.

Now we find that GLP-1 stimulates the growth of new β-cells, the neogenesis of β-cells derived from the progenitor cells located in the ducts of the exocrine pancreas. GLP-1 also stimulates the expression of the transcription factor IDX-1 that appears to be responsible for the initial differentiation of precursors into β-cells and to regulate the expression of the insulin gene.

Thus, we now have evidence that GLP-1 stimulates both the formation of new β-cells and stimulates existing β-cells to grow.

Based on this new evidence, GLP-1 holds promise as a treatment for both type 1 and type 2 diabetes, because in both types of diabetes the progenitor cells in the ducts are unaffected and can be encouraged to develop and grow, and to restore the loss of β-cells mass by the administration of GLP-1.

We have patents on GLP-1 and IDX-1, but it is unclear whether they address this newly discovered property of GLP-1 to induce IDX-1 and thereby to stimulate the growth of β-cells and the production of insulin.

Massachusetts General Hospital

Date: 18-Nov-1997 11:03am GMT
From: Glass, David J.
Glass.David@MGH.HARVARD.EDU@I◊
Dept:
Tel No:

TO: Habener, Joel F (HABENERJ@A1)

Subject: New Invention (New reference MGH 1277.0)

Joel:

I've reviewed the new invention you sent me at the end of October. I agree that it seems to overlap somewhat with your earlier patents, but it is not clear whether this new use would be patentable with respect to the earlier patents. I'm happy to send the new invention to one of our attorneys to answer this question, but I think there is a different threshold question. The new invention is a new use for GLP-1, and as such, can only be practiced by someone licensed under our GLP-1 patents (is this correct?). If so, that means we effectively have only one potential licensee for this new invention, the Scios/Novo "team", and we should contact them about this new use before going too far down the road. Have you told anyone at either company about this new finding?

Let's discuss how best to proceed. Thanks.

David

---------- ATTACHMENT ----------

Massachusetts General Hospital
13th Street, Building 149, Suite 1101
Charlestown, MA 02129
Tel.: 617-726-8608
Fax.: 617-726-1668

Office of Technology Affairs

To: DR. Joel Habener Fax: 6-6954

Company:

CC: Fax:

From: David Glass Date: 7/1/98

Re: New Invention Disclosure

Page(s): including cover sheet 2

☐ Urgent ☐ For Review ☐ Please Comment ☐ Please Reply

• Comments:

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The Massachusetts General Hospital
Thirteenth Street, Building 149, Suite 1101
Charlestown, Ma 02129

DAVID J. GLASS, Ph.D.
Associate Director for Patents
Office of Technology Affairs

Telephone: (617) 726-5474
Telefax: (617) 726-1668
E-mail: glass@helix.mgh.harvard.edu

July 1, 1998

BY FACSIMILE

Michele Cimbala, Esq.
Sterne, Kessler, Goldstein and Fox
1100 New York Avenue, Suite 600
Washington, DC 20005-3934

Re: New Invention Disclosure
Title: Stimulation of IDX-1 Expression and β-Cell Neogenesis by GLP-1
Inventor: Joel Habener, M.D.
MGH Ref: 1277.0

Dear Michele:

Enclosed please find the above-captioned invention disclosure from Dr. Joel Habener, along with some e-mail correspondence from last fall and this week describing the invention and some current research of Dr. Habener's and others' that may be relevant thereto.

It is not clear to me from this correspondence exactly what has been published by other groups (Dr. Habener has evidently not yet published his own research). However, Dr. Habener would like to explore the possibility of filing a new patent application on this invention, either as a stand-alone patent application or as a CIP (or even a continuation or divisional?) to the MGH 213 series of cases that is still pending. Please briefly review this material, talk to Dr. Habener as may be necessary, and let me know what you think our options are with respect to a possible new patent filing. It is not necessary to do a search at this time, although a major concern for me is the impact of our prior patent applications on the patentability of this invention, and whether this might require that we claim priority from these earlier cases.

Thank you very much. Please let me know if you have any questions.

Sincerely,

David J. Glass, Ph.D.

Enclosure
cc: Joel Habener, M.D.

MASSACHUSETTS
GENERAL HOSPITAL

HARVARD
MEDICAL SCHOOL

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Joel F. Habener, M.D.
Professor of Medicine
Harvard Medical School
Chief, Laboratory of Molecular Endocrinology
Massachusetts General Hospital

Tuesday, April 18, 2000

COPY

Marvin C. Guthrie, JD
Executive Director for Patents and Licensing
Office of Corporate Sponsored Research and Licensing
CNY 149.1101

RE : GLP-1

Dear Marvin:

Since the initial submission to you of the extended invention disclosure (October 28, 1997, enclosed), there has been substantial new discovery on the actions of GLP-1 agonists to promote the growth of new pancreatic β-cells and thereby to promote the production of insulin, relevant to the treatment of individuals who have diabetes mellitus due to a failure of the pancreas to produce insulin in amounts sufficient to meet the body's needs.

The novel findings are that GLP-1 stimulates pancreatic stem cells to differentiate into insulin-producing β-cells and does so by stimulating the expression of the homeodomain transcription factor IDX-1 (also known as IPF-1 and PDX-1). GLP-1 is in development for the treatment of diabetes by Novo Nordisk, Eli Lilly, Glaxo Welcome, Novatis, and Bayer. The first generation GLP-1 drugs are scheduled for marketing in late 2002 – early 2003. The MGH holds 4 – 5 patents on GLP-1 for the treatment of diabetes, all of which expire in 2003, 17 years since the filing of the parent application in 1986. This is the time to file a new patent to extend coverage for GLP-1. The estimated worldwide sales of GLP-1 in 2003 are $0.5 – 3.0 billion, depending upon market penetrance to displace the use of insulin. This could represent considerable royalty income to the MGH, if the MGH is appropriately positioned with regard to patent rights.

At present, Stern, Kessler, Goldstein and Fox are dealing with the GLP-1 patents. Banner Witkoff is dealing with the patents on IDX-1 and stem cells. I propose that the OTA devise a strategy to combine these efforts to construct a new patent to propose GLP-1 stimulates pancreatic stem cells to differentiate into insulin-producing β-cells and does so by stimulating the expression of the β-cell-specific master regulator of pancreas development and of insulin gene transcription, IDX-1.

The new data relevant to the construction of a new patent application are:

1. GLP-1 agonists stimulate the formation of new β-cells in the rat *in vivo* and prevent the occurrence of diabetes in the rat model of type 2 diabetes of partial pancreatectomy (Xu et al., Diabetes 48:270,1999). GLP-1 agonists stimulate the expression of IDX-1 (Stoffers et al., Diabetes, in press, May 2000). Pancreatic islets contain pancreatic stem cells (Diabetes, in revision). Pancreatic duct cells contain stem cells that can become β-cells (Peck et al., Nature Medicine, April 2000). Delivery of IDX-1 to liver converts hepatic stem cells into β-cells that produce insulin (Ferber et al., Nature Medicine, May 2000). GLP-1 converts transformed human fetal islet cells into β-cells (Levine et al., in press).

Much of this new fast-breaking information is in press. It is critically important to file a new patent application on GLP-1. I hope to receive a response from OTA ASAP.

Sincerely yours,

Joel F. Habener, M.D.

cc: David J. Glass, John T. Potts, Jr.

JFH/ral

EXHIBIT H

[^{3}H]TdR Labeling of Islet Cells #2

Target cells

~~RIN 1046-38 cells~~ BTC-1 cells
~~HIT cells~~ IEC cells
Duplicates of everything
Really confluent and not so confluent

A. Conditioned media

~~RIN 1056A~~
HAM INRIG9 - 24 hr. media from relatively confluent plates

Run target cells overnight in 0.25% BSA and duplicate wells of cells in FBS (regular) media

Remove media and add either:

1. Conditioned media
2. Normal fresh FBS media
3. 0.25% BSA media
   24 hours later add:
   0.1 mCI (0.1 ml) ^{3}H TdR/ml
   ? 0.05 mCI (0.05 ml) for 1 hr.

B. GLP's

GLP-I(7-37)10^{-7}M
GLP-II(1-33)10^{-7}M
Run target cells overnight in 0.25% BSA and duplicate wells of cells in FBS media

Remove media

Add back BSA media with:

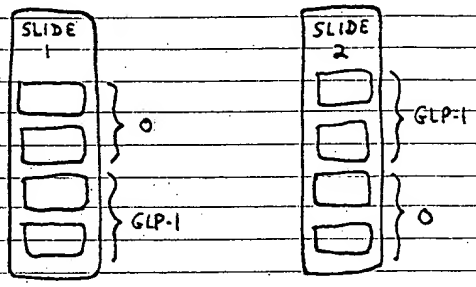
1. Nothing
2. GLP-I(7-37)10^{-7}M
3. GLP-II(1-33)10^{-7}M
4. + regular media (No BSA)

About 24
~~At least 12~~ hours later add 0.1 mCi/ml [^{3}H]TdR for 1 hr.

^{3}H TdR labelling of 1046-38 cells - 1st Attempt. July 16, 1987

GLP-1 (7-37) 10^{-7}M vs O

Setup on Slides:



Slides plated out 1ml/well at a 1→4 dilution on 7/12/87. Plastic slides - 4 well ea. RIN 38 F_3

7/16/87 2:00pm - medium changed to 0.5% FBS to slow growth

7/17/87 8 am - Transfered cells to Medium with 0.25% BSA (no FBS) with/without GLP-1 (7-37) at 10^{-7}M

2:00pm - Added ^{3}H TdR If 1mCi/ml (The way it comes), add 200 λ/well (removed 200 λ medium first) 400 λ / big well

3:00pm - Drained wells by upending on a blue pad, then peeled off wells and washed cells in fresh PBS.

3:15pm - Cells float off slides in whole sheets. Attempt to save by fixing with 4% PFA. Total mess.

Revised Strategy: Grow on glass slides?

Wash cells and fix before ripping off wells.

Fix before washing?

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3H-TdR LABELING OF 1046-38 CELLS

1. Grow cells on slides:

   3 control--media alone

   changed 9/22/87 JFH

   3 experimental-GLP-I(7-37) 10-8 M

   2 experimental-GLP-I(7-37) 10-7 M

2. Transfer to media containing 0.5% FBS and grow (incubate) for 24 hrs.

3. Add GLP-I(7-37) at 0, 10-8, 10-7 M in bioassay buffer), 0.25% BSA.

4. Incubate 6-8 hours.

5. Add 3H-TdR ca. 0.1 mCi per 1-2 ml. Incubate 1 hr.

6. Remove 3H media, wash wells well, and fix for autoradiography.

^{3}H TdR Labelling of 1046-38 and HIT cells

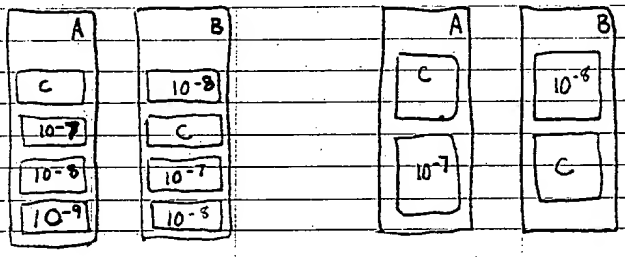
November 10, 1987

11/9/87 11pm Took 2 plates (100mm) HIT F_{18} and 2 plates Jan's 1046-38 F_6 and pooled each set → 50.

I created
- 2 4 well plastic slides at 1→2.5 dilution 1ml/well
- 2 " " 1→10 dilution "
- 2 4 well glass slides 1→2.5 ml "
- 2 " " 1→10 "
- 2 2 well plastic slides 1→2.5 2ml/well
- 2 " " 1→10 "
- 2 2 well glass slides 1→2.5 "
- 2 " " 1→10 "

11/11/87 10 am Changed all media to 0.5% FBS to slow growth – Total volume = 64 ml DMEM
64 ml RPMI

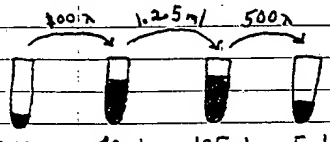
11/12/87 4pm Made up GLP-1 solutions (7-37) and added to cells.
(all in 0.25% BSA + Medium)



40 ml Medium 0.25% BSA
=

						Totals Needed		
2 ml	Control	× 4 = 8	4 ml	Control	× 4 = 16	24 ml	Control	~~24 ml~~
2 ml	10^{-7}	8	2 ml	10^{-7}	8	16 ml	10^{-7}	~~8~~
3 ml	10^{-8}	12	2 ml	10^{-8}	8	20 ml	10^{-8}	~~12~~
1 ml	10^{-9}	4				4 ml	10^{-9}	~~4~~ 2
						Need half in each medium		~~24 ml~~

Dilution Scheme: 0.5ml H_2O to tube = 10^{-5}



Sort of botched makeup of solutions, i.e. ran out so had to make more. Should all be the same, as I was compulsive about it. Put only one ml on 2-well plate wells. 1046-38 cells went first, so ironed the glitches for the HIT cells

11/12/87 11:00pm Had limited Thymidine Resources so added to best guess pairs first – ie plastic 4-well 1→10 dilution slides. Saved medium and used on other slides. 1hr incubation.

Thymidine comes 1mCi/1ml aqueous solution, so added 100λ/well for ~0.1mCi/ml. Swished up pipette to mix.

12:30 Put ^{3}H TdR medium on plastic 4-well 1→2 dilution slides. (Washed first set in PBS 3×10 min. 4% PFA 10 min.)

2:00 Split medium in half – did both sets glass 4-well slides (1→2 and 1→10 dilution 550λ each – figured concentration count.

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^{3}H TdR Labelling of 1046-38 and HIT cells, Continued — November 12, 1987

2:00 am - Generalized mess.

1046-38 cells on first batch of slides mostly vanished. HIT cells clung beautifully. (The 4-well plastic 1→10 dilution slides) Rinsed with H_2O + dried. Then peeled off wells.

So, on the 2nd batch of slides, I was extremely careful (4-well plastic 1→2.5 dilution). I decided to go for the 4% PFA after 2 quick PBS washes. Instantaneously, the 1046-38 cells lifted off in sheets. So I carefully removed the 4% PFA and rinsed only with several drops of H_2O and drained and air dried. I saved some sheets, but the background may be terrible. Preservation looks OK so far. I figure each slide is internally controlled, so if I at least do the same thing to each well in a slide, I can vary a bit between slides. HIT clung beautifully, even though very confluent.

The third batch (all the 4-well glass slides) "A" slides got their peptide medium saved (in case we want to check GLP-1 concentrations). Then I put ½ ml of the hot medium in each well so I could do both the 1→2.5 and 1→10 dilution slides at once. Need to save time. I tried the quickie 4% PFA fix after a fairly long PBS soak session (as long as cells stayed on I figured I should keep rinsing. But then after fixing, the cells were loose and when the slides dried (1046-38) the cell slush gathered in the middle of the slide.

The 4th batch (glass 2-well 1→10 dilution slides) I thought I would try not fixing. When I rinsed quickly with H_2O, the cells plumped up and popped and what was left fell off the slide. When I dried directly from PBS, the salt made them shrivel. I fixed the HIT cells very quickly in 4% PFA and rinsed with H_2O. Some not fixed enough popped. (1046-38)

The 5th batch (glass 2-well 1→2.5 dilution slides) I thought I'd try acetone fixation (it dissolves plastic). It precipitated the salt into a white wasteland.

9:00 am — The remaining slides I was too damn tired to deal with so I let them rot. There were 4: (no great loss) tossed later.

1046-38 1→2 A 2 well plastic
1046-38 1→2 B 2 well plastic
HIT 1→2 A 2well plastic
HIT 1→2 B 2 well glass ← previously grabbed wrong slide.

Conclusions: Rinsing before peeling wells off is a good idea. Can at least contain loose sheets of cells and have a fighting chance to save some. Doesn't prevent 1046-38 cells from coming off in the first place. The 1046-38 cells seem to stick a tad better on glass

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^{3}H TdR Experiments

(1) Further attempts to slow cells down
(2) Further pursuit of "conditioned" media effect

(1) Try growing βTC-1 & IEC-18 cells in 1% FBS for 24 & 48 hrs followed by 1 hr pulse label with ^{3}H TdR
For control use 10% FBS media

on βTC-1 cells
(2) Repeat conditioned media Exp 1 using
Media from INR1G9 again
and media from αTC-1 cells.
and media from βTC-1 cells (homologous media

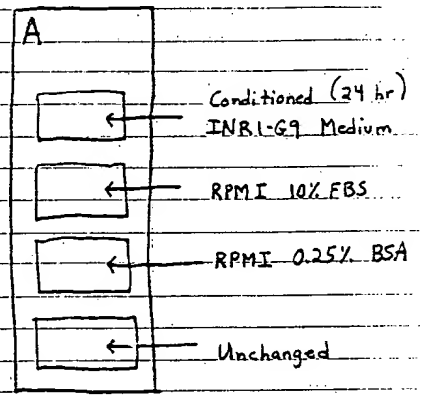
^3H TdR Labeling

4/10/88

Experiment A: Conditioned Media

Slide #	Cell Type	Confluency	RPMI 1st Night
1, 2	β TC-1	full	0.25% BSA
3, 4		½	↓
5, 6		full	10% FBS
7, 8		½	↓
9, 10	IEC-18	full	0.25% BSA
11, 12		½	↓
13, 14		full	10% FBS
15, 16		½	↓

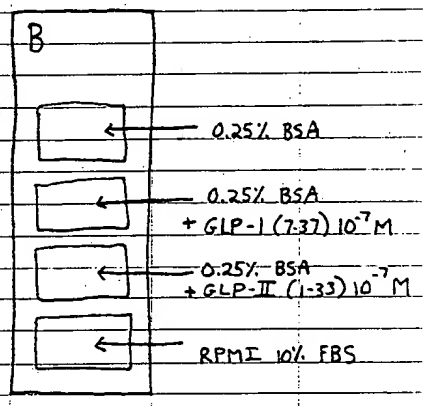
2nd Night (24 hrs)



Experiment B: GLP's

Slide #	Cell Type	Confluency	RPMI 1st Night
17, 18	β TC-1	full	0.25% BSA
19, 20		½	↓
21, 22		full	10% FBS
23, 24		½	↓
25, 26	IEC-18	full	0.25% BSA
27, 28		½	↓
29, 30		full	10% FBS
31, 32		½	↓

2nd Night (24 hrs)



LITHOGRAPHED IN U.S.A. ADDISON-WESLEY PUBLISHING COMPANY, INC., READING, MASS. AW Dataform " 10PL.V

^3H TdR Labeling

4/10/88

Solutions:

500 ml RPMI (2/25)

↓ add 5ml P/S

200 ml	300 ml
↓	↓
add 0.5g BSA	add 30 ml FBS
↓	↓
filter 0.22μ	filter 0.22μ → don't need to do all, just 200 ml or so.

(Use also on INR1-G9 cells for conditioning)

GLP-1 (7-37) 10^{-7} M : Add 0.5 ml H_2O to tube to get 10^{-5} M. Take 200 n → 20 ml BSA medium = 10^{-7} M

~~Take 200 n → 20 ml FBS medium = 10^{-7} M~~

GLP-II (1-33) 10^{-7} M : Add 50 n 10% HAc to tube. Dissolve. Add 450 n H_2O. Take 200 n → 20 ml BSA medium. pH w NaOH = 10

~~Take 200 n → 20 ml FBS medium pH w NaOH = 10^{-7}~~

4/10/88 9-10pm changed slides from BSA or FBS RPMI to test medium. Most slides totally confluent or over confluent. So much for worrying they wouldn't be confluent enough.

4/11/88 11 pm Added ^3H TdR (50 n/ml of a 1mCi/ml = 0.05 mCi/ml = OK by Joel) 32 slides × 4 wells = 128 × 50 = 6.4 ml

(16 slides × 4 wells) + (16 slides × 3 wells) = 5.6 ml

Concentration is what counts. Remove 0.5 ml from each well, add 25 n ^3H TdR to what's left. Shake well. (Irritating habit of medium getting sucked out of end wells by capillary action by cover) Did 5 min / 8 slides

After 1 hr, quickly drained wells with repeater pipette and pipetted in (cold) PBS. 0.5 ml (Did 8 slides × 4 racks in 20 min)

Then drained PBS and added PBS + 4% PFA (0.5 ml) after 20 min. After PFA for 20 min, slides were drained and dismembered. Slides were washed 20 min in 3X changes ICC PBS, then briefly dipped in H_2O to desalt before air drying. Of course morphology declined upon drying, but I didn't lose cells.

4/15/88 Did 6 hr exposure of odd slides with ultrafilm. 4 min dev. 5 min fix. 30 min wash. Air dry. Looked OK.

4/17/88 Did 12 hr NTB-2 (1:1 H_2O) dip of Slide #1, 4 min dev, 5 min fix, 30 min wash, 2½ min counterstain, glycergel mount. Perfect.

LITHOGRAPHED IN U.S.A. ADDISON-WESLEY PUBLISHING COMPANY, INC. READING, MASS A-W Intafoil 10PLY

H

On re thinking it would be good to starve the βTC-1 cells in NO Serum & another batch in 1% Serum for 48 hrs to slow them down

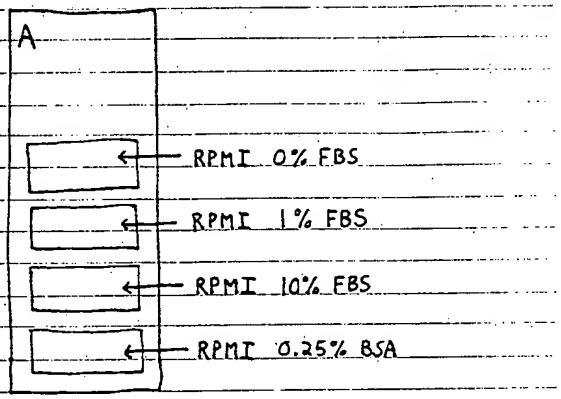
L

^{3}H TdR Labeling

5/15 → 5/19/88

Experiment A: Slowing Cell Growth

Slide #	Cell Type	Confluency	Time
1,2	β TC-1	High	48 hrs
3,4		Medium	
5,6		Low	
7,8		High	24 hrs
9,10		Medium	
11,12		Low	
13,14	IEC-18	High	48 hrs
15,16		Medium	
17,18		Low	
19,20		High	24 hrs
21,22		Medium	
23,24		Low	



5/15 Sunday night: Plated out all the slides.
βTC-1: 2½ 150mm Semiconfluent → 43 ml → 6 slides 1ml/well
Then 6 slides ½ml/well + ¼ ml medium, doubled vol. left → ½
IEC-18: 3/10 100mm plate → 30ml → 4 slides 1ml/well + 4 slides ½ml/well
Then doubled remaining volume → 4 slides ½ml/well

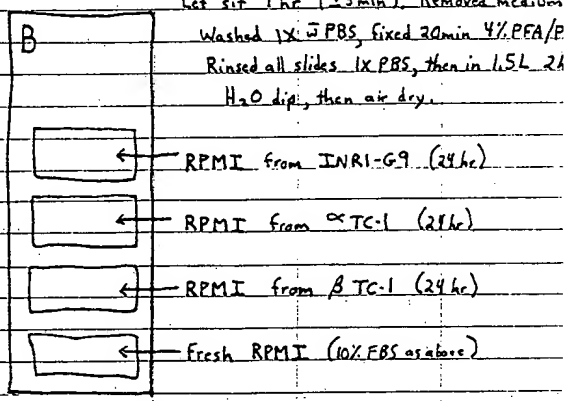
Tuesday Noon: Washed 48 hr slides 2X w/ 0% FBS, then set up, ¾ml/
Put medium on donor plates - ∝, β cells 150mm, INR1G9 100

Wednesday Noon: Washed + Set up rest of slides, ¾ ml/well. Didn't spin donor meds (removed 3

Thursday Noon: Added ^{3}H TdR. (Added 500 x H_2O to 2.1ml left) 20 x/well in 40 in NEN vial.

Experiment B: The Conditioned Medium Effect

Slide #	Cell Type	Confluency	Donor Confluency
25	β TC-1	High	High
26		Medium	
27		Low	
28		High	Low
29		Medium	
30		Low	



Let sit 1 hr (±3 min). Removed medium
Washed 1X w PBS, fixed 20min 4% PFA/P
Rinsed all slides 1X PBS, then in 1.5L 2
H_2O dip, then air dry.

Films: Ultrafilm 9 hrs (forgot) = too long.

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Making Solutions for ^3H TdR

Needed :

24 ml	RPMI 0% FBS + 120 ml to Wash everything	→ Say 400 ml	= 2 bottles filter sterilize everything (Includes Pen/Strep).
24 ml	RPMI 1% FBS	→ Say 50 ml	
24 ml	RPMI 10% FBS + 105 ml for 24 hr collection	→ Say 500 ml	
24 ml	RPMI 0.25% BSA + 24 ml for Exp. B	→ Say 50 ml	
3 ml	RPMI from confluent INRI-69		
3 ml	RPMI from semi-confluent INRI-69		
3 ml	RPMI from confluent α TC-1		
3 ml	RPMI from semi-confluent α TC-1		
3 ml	RPMI from confluent β TC-1		
3 ml	RPMI from semi-confluent β TC-1		

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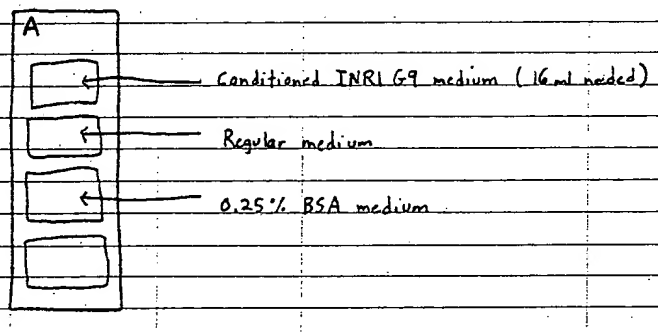
^{3}H TdR Labeling

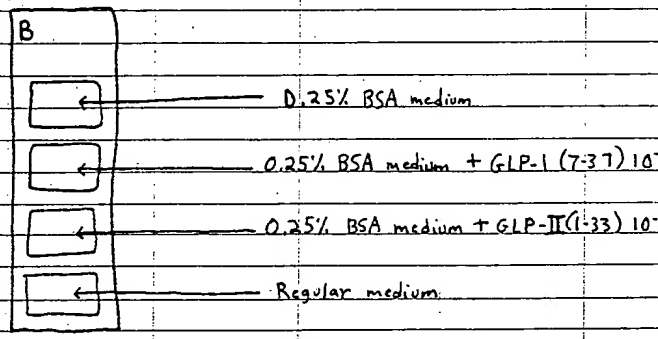
1. Grow up INR1-G9 cells to use medium (RPMI + 10% FBS)
2. Grow up 16 4-well glass slides each of βTC-1 cells (DMEM 15% HS, 2.5% FBS) (can use RPMI 10% FBS)
   IEC cells (RPMI 10% FBS)

   Half the slides should be confluent, half semi confluent (ie 8 each)
   1 ml medium / well
3. Change the medium. Replace half the slides with regular medium, half with 0.25% BSA medium.
4. Let incubate overnight
5. Remove medium from wells. Divide slides into 2 identical piles

   Replace medium from half the slides as follows:



   Replace medium from 2nd half of slides as follows:



6. After 24 hrs incubation, add 50 λ containing 0.1 mCi ^{3}H TdR to each well (in H_2O). Mix with P-1000 pipette.
7. Incubate 1 hr 37°C.
8. Rinse wells several times with PBS. (As much as you can stand without losing too many cells.)
9. Add 4% PFA in PBS to each well. Let sit 16 min (stagger slides 1 min intervals). Rinse in PBS, then peel off wells, quick H_2O dip, air dry.

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